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published in
Journal of Biological Chemistry
2007

DOI (link to publisher)
10.1074/jbc.M706391200

document version
Publisher's PDF, also known as Version of record

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 52, pp. 37875–37884, December 28, 2007

A Novel Secondary Acyl Chain in the Lipopolysaccharide of *Bordetella pertussis* Required for Efficient Infection of Human Macrophages*

Received for publication, August 2, 2007, and in revised form, October 22, 2007 Published, JBC Papers in Press, October 29, 2007, DOI 10.1074/jbc.M706391200

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Lipopolysaccharide is one of the major constituents of the Gram-negative bacterial outer membrane and is a potent stimulator of the host innate immune response. The biosynthesis of the lipid A moiety of lipopolysaccharide is a complex process in which multiple gene products are involved. Two late lipid A acyl transferases, LpxL and LpxM, were first identified in *Escherichia coli* and shown to be responsible for the addition of secondary acyl chains to the 2′ and 3′ positions of lipid A, respectively. Here, we describe the identification of two *lpxL* homologues in the genome of *Bordetella pertussis*. We show that one of them, LpxL2, is responsible for the addition of the secondary myristate group that is normally present at the 2′ position of *B. pertussis* lipid A, whereas the other one, LpxL1, mediates the addition of a previously unrecognized secondary 2-hydroxy laurate at the 2 position. Increased expression of *lpxL1* results in the appearance of a hexa-acylated lipopolysaccharide form with strongly increased endotoxic activity. In addition, we show that an *lpxL1*-deficient mutant of *B. pertussis* displays a defect in the infection of human macrophages.

Pertussis or whooping cough is a severe acute respiratory illness that is characterized by paroxysmal coughing and a distinctive "whooping" sound when air is subsequently inhaled. The disease is highly contagious and most severe in neonates and children younger than one year. Pertussis is caused by the Gram-negative bacterium *Bordetella pertussis*. While the genus *Bordetella* currently encompasses nine species, apart from *B. pertussis* only three other members, *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella holmesii*, have been associated with respiratory infections in humans and other mammals (1). The Gram-negative bacterial cell envelope is composed of two membranes, the inner and the outer membrane, which are separated by the periplasm. The inner membrane is a symmetrical bilayer composed of phospholipids, whereas the outer membrane is asymmetric and consists of phospholipids in the inner leaflet and lipopolysaccharide (LPS)[2] in the outer leaflet. LPS, which is also known as endotoxin, consists of three distinct structural domains: lipid A, the core, and the O-antigen (2). The first domain, lipid A, functions as a hydrophobic membrane anchor and forms the bioactive component of the molecule (3). The structure of lipid A is conserved among different bacterial groups, indicating its importance for the correct functioning of the outer membrane. Generally, lipid A consists of a β-1′,6-linked D-glucosamine (GlcN) disaccharide carrying ester- and amide-linked 3-hydroxyl fatty acids at the C-2, C-3, C-2′, and C-3′ positions, and phosphate groups at positions C-1 and C-4′. The endotoxic activity of LPS is based on the recognition of lipid A by the TLR4/MD-2 complex of the host, which leads to the activation of NF-κB and, consequently, to an increased production and secretion of proinflammatory cytokines, such as IL-6, tumor necrosis factor-α, and IL-1β (4).

Current knowledge about lipid A biosynthesis is mainly derived from studies in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*), where the biosynthetic pathway has been completely elucidated. It consists of nine enzymes that work in a successive order. In the first step, an acyl chain is transferred from the *R*-3-hydroxytetradecanoic acid (3OH C14)-acyl carrier protein to the GlcN 3 position of UDP-N-acetyl glucosamine (GlcNAc) by the acyltransferase LpxA (5, 6). Then, the acylated UDP-GlcNAc is de-acetylated by the LpxC enzyme (7), after which LpxD adds a 3-hydroxyl acyl chain at this position (8), resulting in a UDP-2,3-diacylGlcN molecule. Next, UMP is removed from a proportion of the UDP-2,3-diacylGlcN pool by LpxH (9), before a tetra-acylated GlcN disaccharide is formed by LpxB (5). After 4′-phosphorylation by LpxK, creating a molecule known as lipid IV_A (10), two 2-keto-3-deoxyoctulosonic acid residues are added by KdtA (11), and finally the secondary acyl chains are added by the late acyltransferases LpxL and LpxM (12, 13).

The late acyltransferase LpxL of *E. coli* was found to be responsible for the addition of a secondary laurate (C12) moiety to the 2′ position of lipid A (12, 14). LpxL homologues have been identified in several other Gram-negative bacteria, including *Haemophilus influenzae* (15), *Neisseria meningitidis* (16), *S. typhimurium* (17), and *Yersinia pestis* (18). The second late

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[2] The abbreviations used are: LPS, lipopolysaccharide; *S. typhimurium*, *Salmonella enterica* serovar Typhimurium; LB, Luria Bertani broth; BG, Bordet-Gengou; MS, mass spectrometry; MS/MS, tandem MS; 3OH C10, 3-hydroxydecanoic acid; 3OH C12, 3-hydroxydodecanoic acid; 3OH C14, 3-hydroxytetradecanoic acid; 2OH C12, 2-hydroxydodecanoic acid; C12, laurate; CFU, colony forming unit; MM6, MonoMac 6; IL, interleukin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

acyltransferase, LpxM, is closely related to LpxL and was initially described as a multicopy suppressor of an *lpxL* mutation (19). In *E. coli*, LpxM is responsible for the addition of a secondary myristate (C14) chain at the 3′ position of lipid A (13). Bacteria with mutations in *lpxL* and *lpxM* harbor underacylated LPS species, which display a reduced activity in stimulating innate immune responses (16, 20–21).

The structure of *B. pertussis* lipid A (Fig. 1) resembles that of *E. coli*. It typically consists of a GlcN disaccharide substituted with 3OH C14 residues at positions 2, 2′, and 3′ via ester or amide linkage and with an *R*-3-hydroxydecanoic acid (3OH C10) residue at the 3 position via ester linkage. A secondary C14 replaces the hydroxyl group of 3OH C14 at the 2′ position (Fig. 1) (22). Limited information on the genetics of *Bordetella* lipid A biosynthesis is currently available, and detailed analyses have only been performed for the acyl transferase LpxA and the 2-keto-3-deoxyoctulosonic acid transferase KdtA (23, 24).

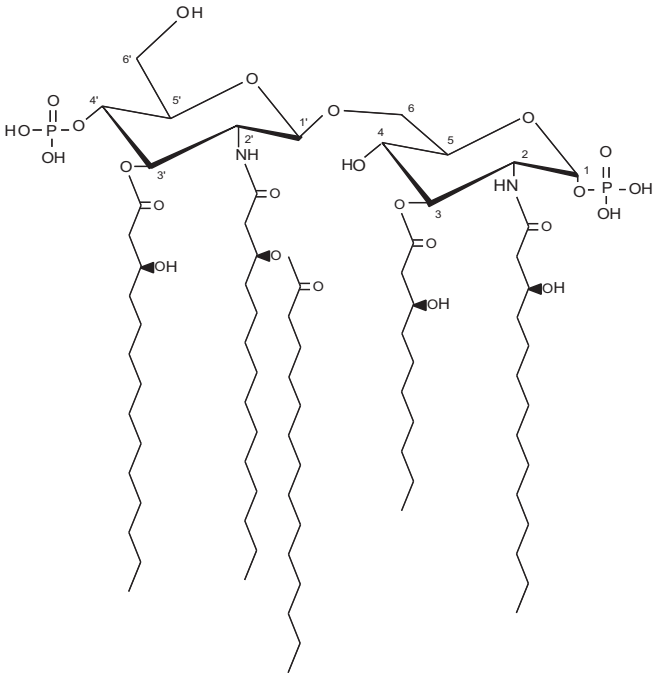

FIGURE 1. ***B. pertussis* lipid A architecture.** *B. pertussis* lipid A consists of a bisphosphorylated GlcN disaccharide substituted with three *R*-3-hydroxytetradecanoic acid moieties and one *R*-3-hydroxydecanoic acid moiety, of which the 2′ fatty-acyl chain is esterified with myristate (22).

The goal of the present study was to identify the gene encoding the enzyme responsible for the attachment of the secondary acyl chain to *B. pertussis* LPS with the eventual goal to inactivate this gene and create a less reactogenic vaccine strain. We identified a locus of two *lpxL* homologues in the genome of *B. pertussis*, which raised the question which of these genes is responsible for the attachment of the single secondary acyl chain and what the function of the other LpxL homologue might be. The study resulted in the identification of new LPS forms in *B. pertussis*, required for successful infection of human macrophages.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—All bacterial strains used are described in Table 1. Typically, the *E. coli* strains were grown at 37 °C in a modified Luria-Bertani broth, designated LB (25), supplemented with 0.2% glucose or at either 30 °C or 42 °C in a synthetic minimal medium (26) supplemented with 0.5% glucose, while shaking at 200 rpm. When appropriate, the media were supplemented with 100 μg/ml ampicillin, 10 μg/ml tetracycline, 10 μg/ml gentamicin, 50 μg/ml nalidixic acid, or 300 μg/ml streptomycin, for plasmid maintenance or strain selection. *B. pertussis* was grown at 35 °C on Bordet-Gengou (BG) agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading). To induce the expression of the *lpxL1* and *lpxL2* genes from plasmids in *B. pertussis*, the bacteria were grown in synthetic THIJS medium (27) supplemented with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 35 °C while shaking at 175 rpm.

TABLE 1
Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Source or reference[b]
B. pertussis		
B213	Str^R derivative of *B. pertussis* strain Tohama	(41)
B213 ΔlpxL1	lpxL1 mutant of B213 strain, Str^R, Gm^R	This study
E. coli		
TOP10F′	*F′{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	F^-, *Δ(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 dlacZΔM15*	(42)
SM10λpir	*thi thr leu fhuA lacY supE recA*::RP4-2-Tc::Mu *λpir* R6K Kan^R	NVI
W3110	Wild-type strain, F^-, λ^-	NVI
MLK53	*lpxL*::Tn10, Tet^R derivative of W3110	(19)
Plasmids		
pCRII-TOPO	*E. coli* cloning vector, Amp^R Kan^R	Invitrogen
pET-11a	*E. coli* high copy expression vector, Amp^R, T7 promoter	Novagen
pMMB67EH	Broad host range expression vector, Amp^R	(43)
pKAS32	Allelic exchange suicide vector, Amp^R	(44)
pBSL141	*E. coli* vector harboring gentamicin-resistance cassette, Amp^R Gm^R	(45)
pLpxL1	pET-11a derivative harboring *B. pertussis lpxL1*	This study
pLpxL2	pET-11a derivative harboring *B. pertussis lpxL2*	This study
pMMB67EH-LpxL1	pMMB67EH derivative harboring *B. pertussis lpxL1*	This study
pMMB67EH-LpxL2	pMMB67EH derivative harboring *B. pertussis lpxL2*	This study
pCRII-LpxL1$_{up}$	pCRII derivative harboring *lpxL1*-upstream sequence	This study
pCRII-LpxL1$_{down}$	pCRII derivative harboring *lpxL1*-downstream sequence	This study
pKAS32-LpxL1$_{KO}$	pKAS32 derivative harboring *lpxL1* knockout construct, Amp^R, Gm^R	This study

[a] Str, streptomycin; Gm, gentamicin; Tet, tetracycline; Amp, ampicillin; Kan, kanamycin.
[b] NVI, Netherlands Vaccine Institute, Bilthoven, The Netherlands.

Recombinant DNA Techniques—All plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega Wizard®Plus Minipreps system. Calf-intestine alkaline phosphatase and restriction endonucleases were used according to the instructions of the manufacturer (Fermentas). DNA fragments were isolated from agarose gels using the Qiagen quick gel extraction kit. Ligations were performed using the rapid DNA ligation kit (Roche Applied Science).

The *lpxL1* and *lpxL2* genes from *B. pertussis* strain B213 were obtained by PCR. The chromosomal template DNA was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95 °C. The suspension was then centrifuged for 1 min at 16,100 × *g*, after which the supernatant was used as template DNA. The sequences of the forward primers, which contained an NdeI site (underlined), including an ATG start codon, were 5′-AACATATGCTCGTCACCCTGTTA-3′ (*lpxL1*) and 5′-AACATATGAGCCAATTCAAGA-3′ (*lpxL2*). The sequences of the reverse primers, which contained a BamHI site (underlined) and included a stop codon, were 5′-AAGGATCCTCATCGTTCGGGTTCCTG-3′ (*lpxL1*) and 5′-AAGGATCCTCAGTACAGCTTGGGCTT-3′ (*lpxL2*). The PCRs were performed under the following condition: 50 μl of total reaction volume, 25 pmol of each primer, 0.2 mM dNTPs, 3 μl of template DNA solution, 1.5% dimethyl sulfoxide, and 1.75 units of Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche Applied Science). The temperature program was as follows: 95 °C for 3 min, a cycle of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C, repeated 30 times, followed by 10 min at 72 °C and subsequent cooling to 4 °C. The PCR products were purified from agarose gel and subsequently cloned into pCRII-TOPO. Plasmid DNA from a correct clone was digested with NdeI and BamHI, and the LpxL-encoding fragments were ligated into NdeI- and BamHI-digested pET-11a. The ligation mixture was used to transform *E. coli* DH5α using the $CaCl_2$ method (28). Plasmids containing the correct inserts were designated pLpxL1 and pLpxL2. The nucleotide sequences of the cloned genes were confirmed by sequencing in both directions.

To allow for expression in *B. pertussis*, the *lpxL1* and *lpxL2* genes were subcloned into the broad host range, low copy number vector pMMB67EH. To this end, pLpxL1 and pLpxL2 were digested with XbaI and HindIII, and the relevant fragments were ligated into XbaI- and HindIII-digested pMMB67EH. The ligation mixture was used to transform *E. coli* DH5α. Plasmids with the correct inserts were designated pMMB67EH-LpxL1 and pMMB67EH-LpxL2 (Table 1). The pMMB67EH-based plasmids were used to transform *E. coli* SM10(λpir), which allowed for subsequent transfer of the plasmids to *B. pertussis* by conjugation. For complementation experiments, *E. coli* strains W3110 and MLK53 (Table 1) were transformed with the plasmids.

To construct a *B. pertussis lpxL1* mutant strain, we amplified a part of the DNA upstream of *lpxL1* from *B. pertussis* strain B213 by using primers 5′-AAATTCGCTCTGGCGCTGCAC-3′ and 5′-AATCAGCACGCGTCTGACCGATGCGAATGAAAGGGCGG-3′, containing an MluI site (underlined). Additionally, a DNA fragment downstream of *lpxL1* was obtained by PCR with primers 5′-AAGTCAGACGCGTGCTGAGACAGCGCGCGGCAGGAACC-3′, containing an MluI site (underlined), and 5′-AATCCACGTGATAGCGCCCGGT-3′. Both PCR products were cloned into pCRII-TOPO, resulting in plasmids pCRII-LpxL1$_{up}$ and pCRII-LpxL1$_{down}$, respectively. An MluI-XbaI fragment of pCRII-LpxL1$_{down}$ was ligated into MluI-XbaI-restricted pCRII-LpxL1$_{up}$. The resulting plasmid was cut with MluI to allow for insertion of the gentamicin resistance cassette from plasmid pBSL141 obtained by MluI digestion. Finally, an XbaI-SacI fragment of the construct obtained was ligated into the XbaI-SacI-restricted suicide plasmid pKAS32. The final construct, designated pKAS32-LpxL1$_{KO}$, contained the gentamicin resistance cassette in the reverse orientation relative to the transcription direction of the *lpxL1* gene and was used to construct a *B. pertussis lpxL1* mutant by allelic exchange. Transformants were screened by PCR using various primer sets.

Isolation and Analysis of LPS—LPS was isolated using the hot phenol/water extraction method (29) with slight modifications (30). The fatty acid composition was analyzed using a 6890 Agilent gas chromatograph (31). The lipid A moiety of LPS was isolated as described (30) and used for structural analysis by nanoelectrospray tandem mass spectrometry (MS/MS) on a Finnigan LCQ in the negative (MS) or positive (MS/MS) ion mode (32).

Stimulation of Macrophages and IL-6 Quantification—The human macrophage cell line MonoMac 6 (MM6) (33) was stimulated with serial dilutions of whole bacterial cell suspensions or purified LPS as described (30). The bacterial cell suspensions were prepared by collecting the cells from cultures by centrifugation, after which they were resuspended in phosphate-buffered saline at an optical density at 590 nm (A_{590}) of 1.0, heat-inactivated for 10 min in the presence of 8 mM formaldehyde, and stored at 4 °C. Following stimulation, IL-6 concentrations in the culture supernatants were quantified with an enzyme-linked immunosorbent assay against human IL-6 according to the manufacturer's instructions (PeliKine Compact^{TM}).

Infection of Human Macrophages—For infection of human macrophages, bacteria were grown for 16 h on fresh BG blood agar plates, after which they were washed once with phosphate-buffered saline and resuspended in 1 ml of Iscove's modified Dulbecco's medium (Invitrogen). Bacteria were added to 5 × 10^5 MM6 cells, which were maintained in 0.25 ml of pre-warmed Iscove's modified Dulbecco's medium in 24-well tissue culture plates, at a multiplicity of infection of 10 (final volume = 500 μl). After 2 h of incubation (5% CO_2 and 37 °C), 100 μg/ml colistin sulfate (end concentration) was added to the wells, after which the plates were incubated further for 2 h at 37 °C. Then, the MM6 cells were collected by centrifugation and washed twice with Iscove's modified Dulbecco's medium, after which they were lysed (1 min at 22 °C) in 0.15 ml of phosphate-buffered saline containing 0.1% Triton X-100. The lysed cells were plated onto BG blood agar plates, and the number of viable intracellular bacteria was estimated by determining the number of colony forming units (CFUs) after 72 h of growth. Infection experiments were repeated three times. The colistin sensitivity of the wild-type and mutant strain was determined by growing them in the presence of various concentrations of colistin and, after

diluting the suspensions and plating them on BG blood agar plates, counting the number of CFUs.

Association of B. pertussis to Human Macrophages—Association of bacteria to MM6 cells was evaluated by flow cytometry. 2×10^9 bacteria were labeled by incubation with 50 μg of fluorescein isothiocyanate in 1 ml of 50 mM carbonate/bicarbonate buffer (pH 9.6) at room temperature for 20 min. The fluorescein isothiocyanate-labeled bacteria were then washed twice with TSA (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% human serum albumin (w/v) (Sigma)), passaged through a 5-μm filter (Millipore) for declumping, and resuspended in TSA to an absorbance at 600 nm of 1. Association to macrophages was assessed in TSA buffer by incubating (45 min at 37 °C or 4 °C) the fluorescein isothiocyanate-labeled bacteria with the macrophages (multiplicity of infection 2, 10, and 50) and, after washing the cells, measuring the percentage of fluorescently labeled macrophages in FL1 by flow cytometry (FACScan, BD Biosciences).

Statistical Analysis—Data were statistically analyzed using two-way analysis of variance followed by Bonferroni's multiple comparison test (GraphPad). Alternatively, a Student's *t* test (two-tailed, two-sample unequal variance) was used. Differences were considered to be significant when $p < 0.05$.

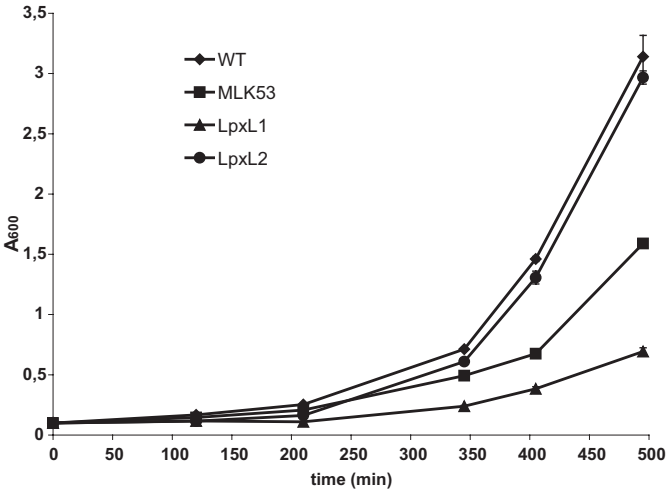

FIGURE 2. **Complementation of the temperature-sensitive growth phenotype of *E. coli lpxL* mutant MLK53.** Bacteria were grown to early log phase in synthetic minimal medium at 30 °C. After 3.5 h, the bacteria were transferred to LB and further incubated at 42 °C. *WT*, wild-type *E. coli* strain W3110 harboring the empty pMMB67EH vector; *MLK53*, *E. coli lpxL* mutant strain harboring the empty pMMB67EH vector; *LpxL1*, *E. coli* MLK53 expressing *B. pertussis lpxL1*; *LpxL2*, *E. coli* MLK53 expressing *B. pertussis lpxL2*. Absorbance was measured at 600 nm. The experiment was performed in triplicate (*error bars* show the standard deviation). A representative result of two independent experiments is shown.

RESULTS

Identification of Late Lipid A Acyltransferase Homologues in B. pertussis—The 306- and 323-amino acid residue sequences of the *E. coli* K-12 LpxL and LpxM proteins with GenBank™ accession numbers NP_415572 and NP_416369, respectively, were used to identify putative *lpxL* and *lpxM* homologues in the complete *B. pertussis* genome sequence present in the NCBI data base (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). BLAST search (34) revealed the presence of two homologues of *lpxL* and *lpxM*, *i.e.* BP3072 and BP3073 with GenBank™ accession numbers NP_881643 and NP_881644, respectively. BP3072 and BP3073 show sequence identities of 21 and 29% to *E. coli* LpxM, respectively, of 23 and 31% to *E. coli* LpxL, respectively, and of 25% to each other. Because both proteins show a higher sequence identity to *E. coli* LpxL than to *E. coli* LpxM, BP3072 and BP3073 were designated *lpxL1* and *lpxL2*, respectively. The open reading frames are adjacent to one another, with the stop codon of *lpxL1* overlapping with the start codon of *lpxL2*, and, therefore, seem to form an operon. Upstream, in the reverse orientation, and downstream of the operon, genes are located putatively encoding a homologue of the *S*-adenosylmethionine synthetase MetK and of the diaminopimelate epimerase DapF, respectively. Further BLAST analysis revealed the presence of *lpxL1* and *lpxL2* homologues in *B. parapertussis*, *i.e.* BPP0191 and BPP0190 with GenBank™ accession numbers NP_882552 and NP_882551, respectively, and in *B. bronchiseptica*, *i.e.* BB0194 and BB0193 with GenBank™ accession numbers NP_886744 and NP_886743, respectively. The mutual sequence identity between the *Bordetella* proteins is 97% for the LpxL1 proteins and 98% for the LpxL2 proteins. Furthermore, the genetic organization of the *lpxL1*/*lpxL2* operon is conserved among the *Bordetella* strains.

Cloning of lpxL Genes and Complementation of the E. coli lpxL Mutant Phenotype—E. coli lpxL mutants show a growth defect on nutrient broth above 32 °C (14). To test whether the identified *B. pertussis lpxL* homologues can complement this phenotype, we cloned the *lpxL1* and *lpxL2* genes into the broad host range, low copy number vector pMMB67EH under the control of the tac promoter and used the resulting plasmids to transform *E. coli lpxL* mutant strain MLK53 (19). As controls, both MLK53 and the parental *E. coli* strain W3110 were transformed with vector pMMB67EH. The strains were first grown to early log phase in synthetic minimal medium at 30 °C, after which the bacteria were transferred to LB and further incubated at 42 °C. The growth defect of the *E. coli lpxL* mutant was complemented by the plasmid harboring *lpxL2* (Fig. 2). The plasmid encoding LpxL1 did not complement the phenotype and its presence, as compared with the empty vector control, seemed to hamper growth even further.

Overexpression of lpxL1 and lpxL2 in B. pertussis—The effect on LPS composition of *lpxL1* and *lpxL2* overexpression was studied in *B. pertussis* strain B213 after introduction of the pMMB67EH-derived plasmids. No obvious effect of *lpxL1* and *lpxL2* overexpression was observed upon Tricine-SDS-PAGE analysis of isolated LPS (data not shown). To evaluate possible alterations in LPS composition in more detail, the lipid A moieties of the strains were analyzed by MS in the negative-ion mode. This analysis revealed the presence of two major lipid A species in wild-type LPS (Fig. 3*A*). The peak at *m/z* 1557 represents the characteristic penta-acylated bis-phosphate species that is typically found in *B. pertussis* (22), whereas the peak at *m/z* 1477 corresponds to a penta-acylated mono-phosphate species. Besides these two major lipid A species, several minor species were detected. The two peaks at *m/z* 1307 and 1251 represent deacylated lipid A species of the molecular ion at *m/z*

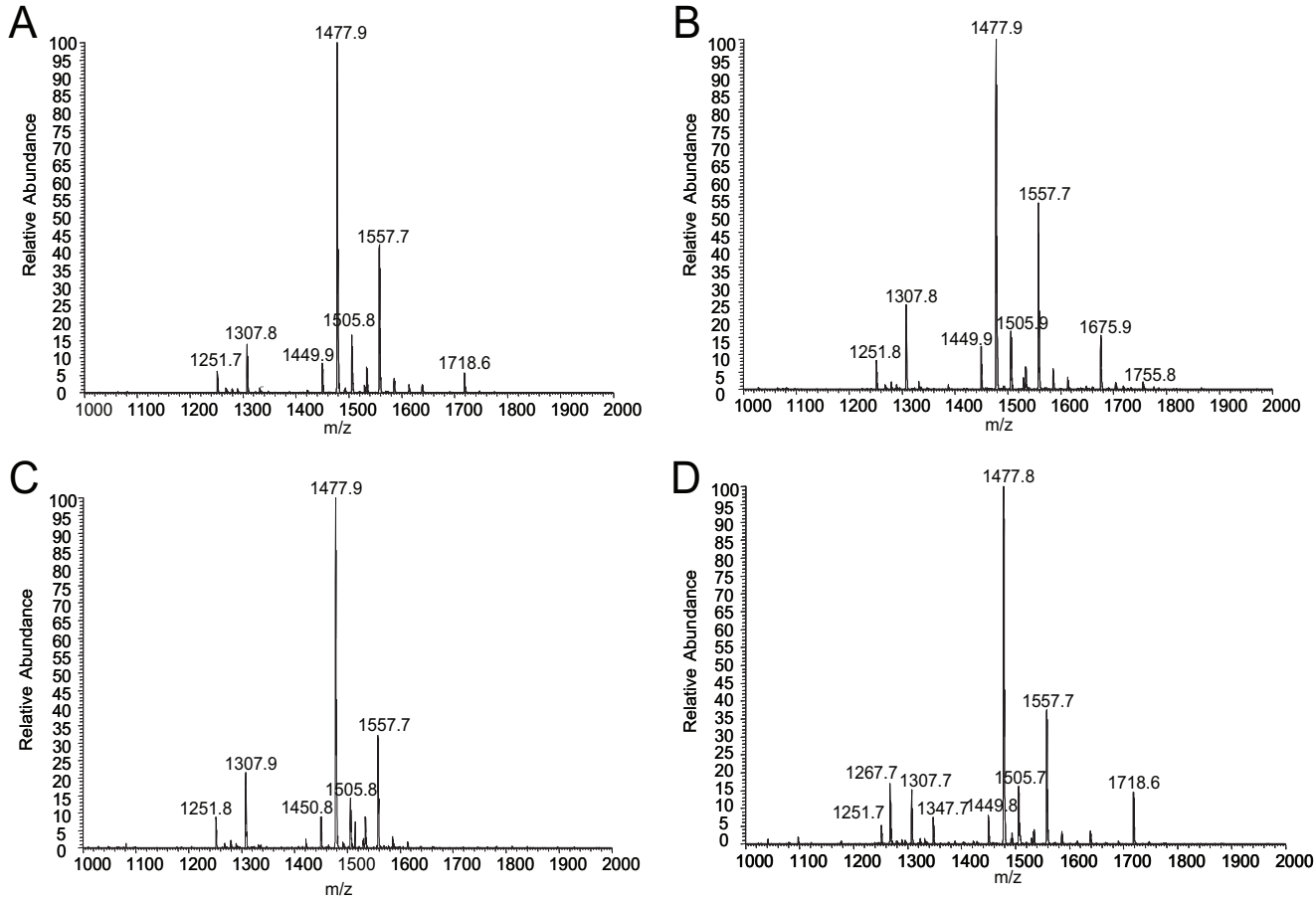

FIGURE 3. **Structural analysis by MS of purified *B. pertussis* LPS.** Lipid A species from wild-type *B. pertussis* strain B213 (*A*) and its derivatives overexpressing *lpxL1* (*B*), *lpxL2* (*C*), or deficient for *lpxL1* (*D*) were analyzed by MS. Peaks at *m/z* 1557, 1477, 1307, and 1251 were interpreted as the characteristic penta-acylated bisphosphate species that is typically found in *B. pertussis*, the corresponding penta-acylated monophosphate species, the deacylated lipid A species of the molecular ion at *m/z* 1477 missing the primary 3OH C10 residue at the 3 position, and the deacylated lipid A species of the molecular ion at *m/z* 1477 missing a primary 3OH C14 residue, respectively. The peaks at *m/z* 1449, 1505, 1676, and 1755 correspond to the molecular ions present at *m/z* 1251, 1307, 1477, and 1557, but containing an extra hydroxyl C12 group (*m/z* 198).

1477 that miss the primary 3OH C10 residue at the 3 position and a primary 3OH C14 residue, probably at the 3′ position (30), respectively. Additional minor species included molecular ions at *m/z* 1449 and 1505. Mass calculations indicate that these species most likely correspond to the molecular ions at *m/z* 1251 and 1307, substituted with an additional hydroxyl C12 chain (*m/z* 198). The peak at *m/z* 1718 represents lipid A modified with hexosamine.[3]

Upon overexpression of *lpxL1* (Fig. 3*B*), clear changes in the spectrum of lipid A species were seen. The new lipid A species detected were peaks at *m/z* 1676 and 1755, which appeared only after overexpression of *lpxL1* (Fig. 3*B*). The mass of these peaks corresponds to the molecular ions at *m/z* 1477 and 1557, respectively, substituted with an additional hydroxyl C12 chain. No obvious differences were observed upon *lpxL2* overexpression (Fig. 3*C*).

To determine the location of the additional hydroxyl C12 chain in the ion at *m/z* 1676, it was subjected to MS/MS analysis in the positive ion mode. As shown in Fig. 4*A*, colliding the ion resulted in multiple fragmentation ions, including ones at *m/z* 904 and 756. These two ions derive from the non-reducing and the reducing half of the lipid A molecule, respectively, with the latter one carrying the additional hydroxyl C12 at either the 2 or the 3 position. This was confirmed by performing the same MS/MS analysis on the wild-type penta-acylated ion at *m/z* 1557, which resulted in corresponding ions at *m/z* 904 and 558, *i.e.* no hydroxyl C12 at the reducing half (Fig. 4*B*). To discriminate between the presence of the C12 hydroxyl chain at the 2 or the 3 position, LPS isolated from the *lpxL1*-overexpressing strain was subjected to *in vitro* 3-*O*-deacylation using purified recombinant PagL enzyme from *Pseudomonas aeruginosa* as described previously (35). MS analysis revealed that 3-*O*-deacylated lipid A species still carried hydroxyl C12 moieties, which unequivocally showed that the hydroxyl C12 acyl chain was present at the 2 position (result not shown). Finally, the fatty acid content of the isolated LPS was analyzed by gas chromatography. The results revealed that LPS from the *lpxL1*-overexpressing strain contained, in contrast to the LPS isolated from the wild-type and *lpxL2*-overexpressing strain, 2-hydroxyl dodecanoic acid (2OH C12), whereas all strains contained minor, but similar amounts of 3-hydroxyl dodecanoic acid (3OH C12) (Fig. 5). The presence of minor amounts of 3OH

[3] J. Geurtsen, M. Dzieciatkowska, L. Steeghs, J. Boleij, K. Boren, G. Akkerman, H. J. Hamstra, J. Li, J. Richards, J. Tommassen, and P. van der Ley, manuscript in preparation.

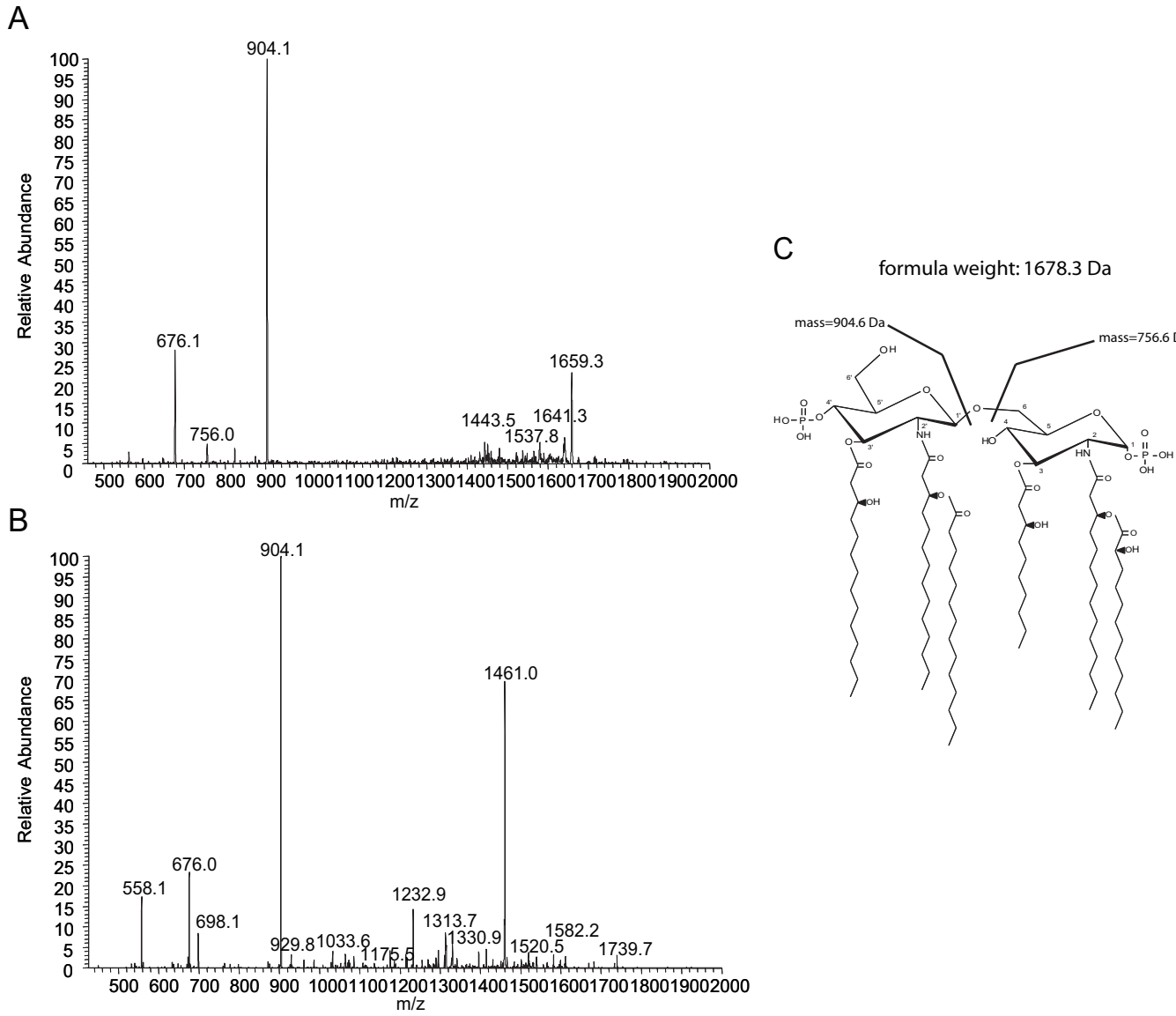

FIGURE 4. ESI-MS/MS analysis of the molecular ion at *m/z* 1676 (*A*) and 1557 (*B*) showing fragmentation ions at *m/z* 904 and 756, and at *m/z* 904 and 558, respectively, corresponding to the proximal and distal parts of hexa- and penta-acylated *B. pertussis* LPS, respectively. *C*, schematic representation of hexa-acylated *B. pertussis* LPS containing a secondary hydroxyl C12 chain at the 2 position.

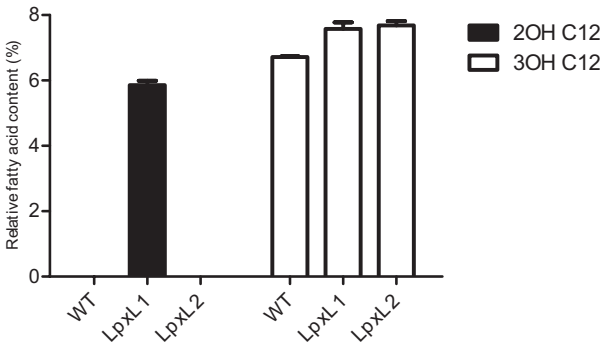

FIGURE 5. **Gas chromatographic analysis of LPS from wild-type, *lpxL1*-, and *lpxL2*-overexpressing *B. pertussis* strains.** LPS purified from wild-type *B. pertussis* strain B213 (*WT*), *B. pertussis* strain B213 overexpressing *lpxL1* (LpxL1), and *B. pertussis* strain B213 overexpressing *lpxL2* (LpxL2) was analyzed by gas chromatography. Indicated are the normalized 2OH C12 and 3OH C12 contents with the 3OH C14 content set at 100. The analysis was performed in triplicate (*error bars* show the standard deviation).

C12 in wild-type *B. pertussis* LPS can be explained by the relaxed acyl chain specificity of the *B. pertussis* LpxA enzyme (23). Overall, these data show that overexpression of *lpxL1* leads to the specific incorporation of 2OH C12 into *B. pertussis* lipid A and that this moiety is located at the 2 position (Fig. 4*C*).

Endotoxic Activity of LPS and Whole Bacterial Cells—To determine whether *lpxL1* or *lpxL2* overexpression affected the endotoxic activity of the LPS, the potency of the purified LPS to stimulate the production of IL-6 by the human macrophage cell line MM6 was tested (Fig. 6). As compared with wild-type LPS, the purified LPS from the strain overexpressing *lpxL1* had a strongly increased potency to stimulate the macrophages, as can be expected from its increased amount of hexa-acyl lipid A (Fig. 6*A*). Similarly, whole cell suspensions of *B. pertussis* cells overexpressing *lpxL1* showed, as compared with *B. pertussis* cells containing the empty vec-

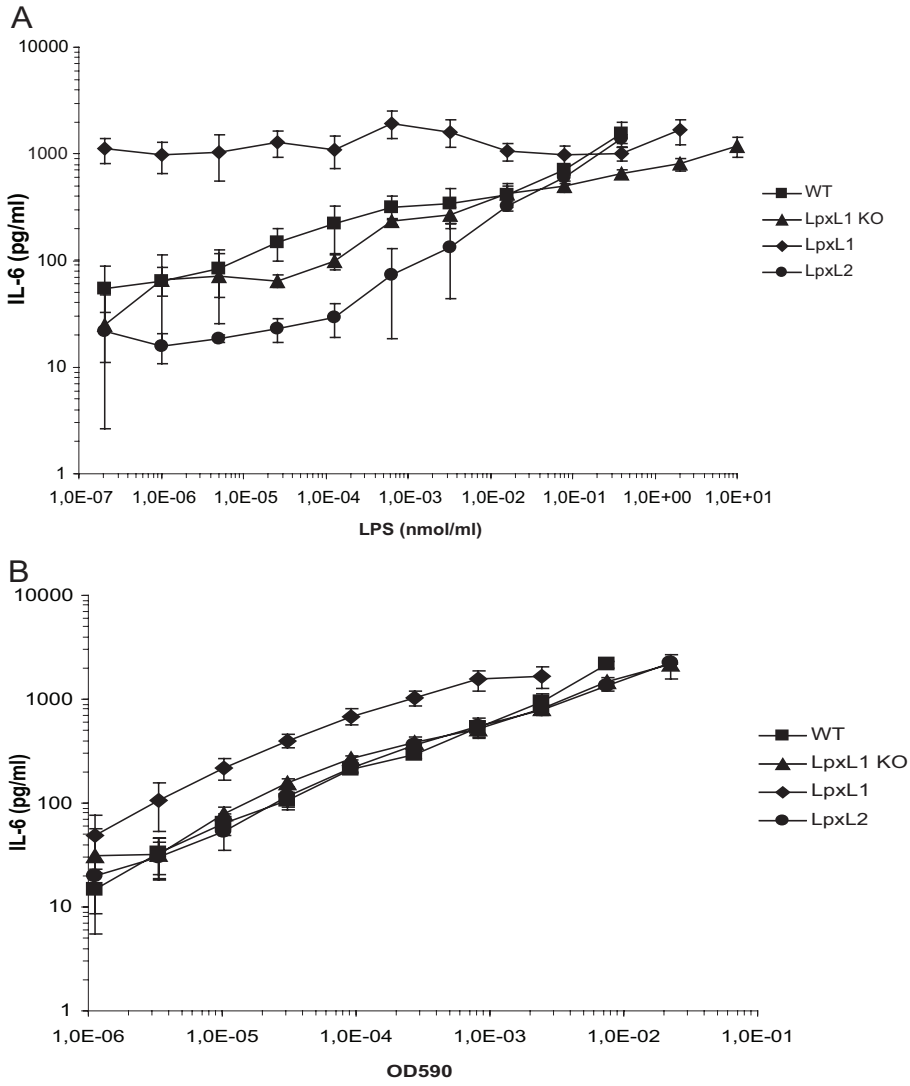

FIGURE 6. **IL-6 induction by purified *B. pertussis* LPS and whole cells.** The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of stock solutions of LPS purified from a wild-type (*WT*), an *lpxL1*- or *lpxL2*-overexpressing, or an *lpxL1*-deficient (*LpxL1 KO*) *B. pertussis* strain (*A*), or a whole cell suspension of *B. pertussis* cells deficient for *lpxL1* (*LpxL1 KO*), overexpressing *lpxL1* or *lpxL2*, or containing the empty vector pMMB67EH (*WT*) (*B*). IL-6 concentrations in the culture supernatant were quantified in an enzyme-linked immunosorbent assay against human IL-6. The data represent the averages of three individual experiments (*error bars* show the standard deviation).

tor, an increased potency to stimulate the macrophages (Fig. 6*B*). Consistent with the MS data, which did not reveal an altered LPS composition, overexpression of *lpxL2* did not significantly alter the endotoxic activity of the LPS or whole bacterial cells.

Construction and Phenotype of a B. pertussis lpxL1 Mutant Strain—One of our initial aims was to create *B. pertussis* mutant strains with less reactogenic LPS. To achieve this goal, we made several plasmid constructs that were suitable for replacing the *lpxL* genes on the *B. pertussis* chromosome by an antibiotic resistance cassette via allelic exchange. Following this strategy, we easily obtained clones in which the *lpxL1* gene was replaced by a gentamicin resistance marker. However, this was not the case for *lpxL2* or the entire *lpxL1*/*lpxL2* operon. We have tried various strategies, using different constructs, different markers, and different selection temperatures, but up till now, we have not been able to isolate a viable *lpxL2* or *lpxL1/lpxL2 B. pertussis* mutant.

To characterize the *lpxL1* mutant obtained, we first studied its growth characteristics. At both 35 °C and at 28 °C, the growth of the mutant strain in synthetic THIJS medium was comparable to that of the wild-type strain (data not shown). Additionally, Western blots showed that the expression of the virulence factors pertactin, fimbriae, pertussis toxin, and filamentous hemagglutinin was unaffected in the mutant strain (data not shown). For structural analysis, LPS was isolated from the mutant strain and analyzed by MS (Fig. 3*D*). The obtained spectrum showed that the LPS content of the *lpxL1*-deficient mutant was highly comparable to that of the wild-type strain. Interestingly, the spectrum of the mutant strain still contained peaks at *m/z* 1449 and 1505, suggesting that these ions represent 3OH C12-substituted and not 2OH C12-substituted LPS species. In addition, two novel LPS species, at *m/z* 1267 and 1347, respectively, were present. These peaks most likely correspond to the molecular ions at *m/z* 1477 and 1557, respectively, but without the secondary C14 chain. The presence of these two additional peaks suggests that inactivation of *lpxL1* leads to a slightly reduced *lpxL2* expression, possibly by a polar effect of the gentamicin resistance marker. We also tested whether the mutant strain would have a decreased capability of inducing the production of pro-inflammatory cytokines, because overexpression of *lpxL1* led to increased stimulatory activity (Fig. 6). However, this was not the case (Fig. 6). All data presented above suggest that under the growth conditions here applied *lpxL1* is not expressed, which could mean that *lpxL1* expression is only beneficial for the bacterium under certain conditions.

Impaired Infection of Human Macrophages by the B. pertussis lpxL1 Mutant Strain—*B. pertussis* is capable of invading and surviving within various eukaryotic cell types, including human macrophages (36). In a previous study, it was shown that an *lpxM* mutant of *Neisseria gonorrhoeae* was impaired in its survival inside urethral epithelial cells (37). To test whether the *B. pertussis lpxL1* mutant is affected in its ability to infect human macrophages, we determined the number of bacteria that could be recovered from the intracellular compartment after 2 h of

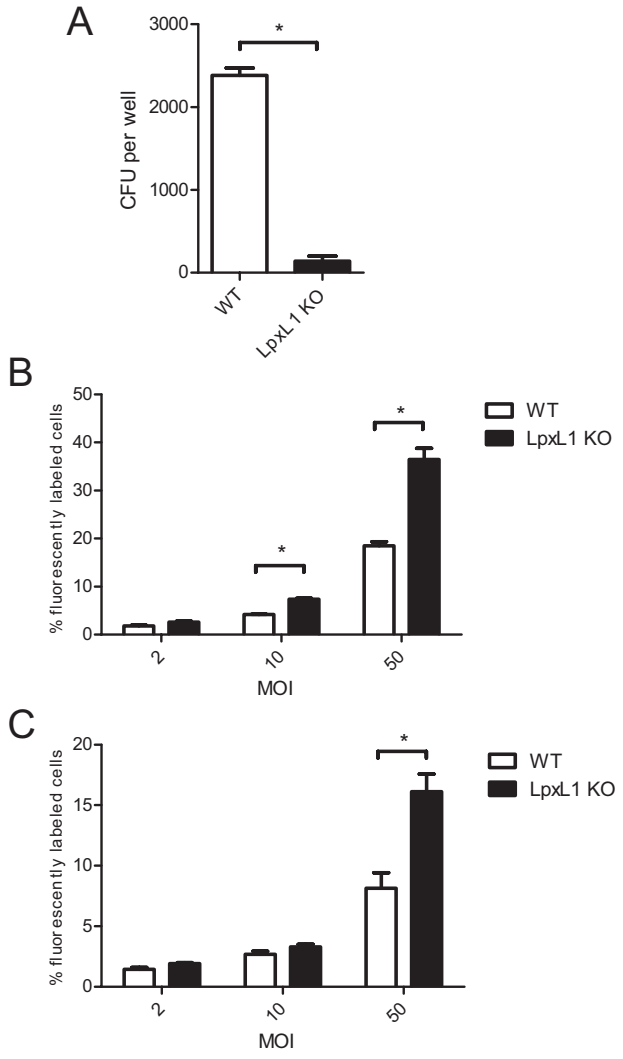

FIGURE 7. *A*, infection of human macrophages by *B. pertussis* wild-type and *lpxL1* mutant strains. Human macrophage cell line MM6 was infected for 2 h with the *B. pertussis* wild-type (*WT*) or *lpxL1* mutant (*LpxL1 KO*) strain. The number of bacteria recovered from the intracellular compartment after killing of remaining extracellular bacteria was determined by CFU counting. The data represent the averages of three individual experiments. *B* and *C*, association of wild-type *B. pertussis* (*WT*) and *B. pertussis* cells deficient for *lpxL1* (*LpxL1 KO*) with human macrophage cell line MM6. Fluorescently labeled bacteria were incubated with the macrophages at 37 °C (*B*) or 4 °C (*C*), and bacterial association was evaluated by flow cytometry. The numbers along the *x*-axis represent the multiplicity of infection; the percentage of fluorescently labeled cells is shown on the *y*-axis. The experiment was performed in triplicate. A representative result of two independent experiments is shown. *Error bars* show standard deviations, and *asterisks* mark significant differences ($p < 0.05$).

infection. However, we first tested whether the wild-type and mutant strain were similar in their ability to survive within medium and in their direct sensitivity to colistin; indeed, no differences in these respects were observed (data not shown). As compared with the wild-type strain (2381 ± 92 CFU/well), the number of bacteria recovered for the *lpxL1* mutant strain was >15-fold lower (138 ± 62 CFU/well) ($p < 0.0001$) (Fig. 7*A*). Thus, apparently, the activity of LpxL1 is required for the efficient infection of human macrophages by *B. pertussis*. To determine whether the decreased infectivity of the LpxL1-deficient strain is due to an abrogated association of the bacteria with the macrophages, we performed a flow cytometric assay in which we assessed the wild-type and the LpxL1-deficient strains for their capacity to associate with the macrophages at either 37 °C (Fig. 7*B*) or at 4 °C (Fig. 7*C*). At both temperatures, the LpxL1-deficient strain showed an increased, rather than a decreased association to the macrophages, thus excluding the possibility that the differences in infectivity are due to a reduced association of the mutant with the macrophages. Therefore, the *lpxL1* mutant is probably deficient in intracellular survival.

DISCUSSION

B. pertussis lipid A has been reported to consist of pentaacylated lipid A species containing four primary hydroxylated acyl chains and one secondary acyl chain, *i.e.* a C14 acyl chain at the 2′ position (22). The goal of this study was to identify and characterize the enzyme responsible for the addition of the secondary C14 acyl chain. BLAST searches using the *E. coli* late acyl transferases LpxL and LpxM as leads identified two homologues in the *B. pertussis* chromosome. This finding raised the questions which of the two *lpxL* homologues in *B. pertussis* mediates the addition of the secondary C14 chain to the LPS and what the function of the other *lpxL* homologue might be. We found that LpxL2, but not LpxL1, was capable of fully restoring the temperature-sensitive phenotype of an *E. coli lpxL* mutant, indicating that the function of *B. pertussis* LpxL2 resembles that of *E. coli* LpxL, and, thus, that LpxL2 mediates the addition of the secondary C14 acyl chain to the 2′ position of *B. pertussis* lipid A. This conclusion was further supported by mass spectrometry and endotoxic activity data, which showed that *lpxL2* overexpression in *B. pertussis* did not lead to significant changes in the composition or toxicity of the LPS. Because already 100% of the *B. pertussis* lipid A species contain a secondary C14 acyl chain at the 2′ position, overexpression of the responsible enzyme was not expected to affect LPS structure or toxicity. The observation that LpxL1 was unable to complement the *E. coli lpxL* mutant phenotype was a first indication that LpxL1 has a different function. We showed that *lpxL1* overexpression in *B. pertussis* leads to the accumulation of a novel, previously undetected lipid A species in *B. pertussis* containing an extra secondary 2OH C12 chain at the 2 position. Thus, either LpxL1 functions as a 2-hydroxyl lauryl transferase, which directly attaches a 2OH C12 moiety to lipid A, or, alternatively, as a regular lauryl transferase, which adds a C12 acyl chain that is later on hydroxylated by a another enzyme, for instance LpxO, for which a homologue has been found in the *B. pertussis* genome (38). Another question is why *lpxL1* expression in *E. coli* MLK53 did not restore the mutant phenotype, because it is known that overexpression of a lipid A-acyl transferase with a different specificity than LpxL, such as LpxM, can also restore the mutant phenotype (19). Possible answers to this question are that *lpxL1* expression in *E. coli* was not high enough to support complementation, that LpxL1 functions only after a secondary acyl chain has been attached to the 2′ position, which is not the case in the *E. coli lpxL* mutant LPS, or that the acyl chain donor was not sufficiently present. The latter possibility is probably only true when LpxL1 indeed directly transfers a 2OH C12 chain.

In the wild-type *B. pertussis* strain, all lipid A species found were secondary acylated with C14, and no species substituted

with secondary 2OH C12 groups were found. Although hydroxylated C12-substituted species were present (Fig. 3*A*, peaks at *m/z* 1449 and 1505), we demonstrated, by showing that these peaks were also present in the LPS from the *lpxL1*-deficient strain, that they most likely correspond to lipid A forms in which the primary 3OH C10 acyl chain or one of the primary 3OH C14 acyl chains is substituted by 3OH C12. These forms must constitute the source of the minor amounts of 3OH C12 detected by gas chromatography in the LPS of all strains (Fig. 5). Thus, apparently, under the growth conditions here applied, LpxL1 activity in the wild-type strain was absent, whereas LpxL2 was highly active. Because *lpxL1* and *lpxL2* almost certainly constitute an operon, this raises an important question as to how the bacterium is capable of controlling LpxL1 activity. One possibility is that *lpxL1* and *lpxL2* expression is differentially controlled at the level of translation initiation. Analysis of the nucleotide sequence upstream of the ATG start codons of *lpxL1* and *lpxL2* supports this hypothesis. The *lpxL2* start codon is preceded by a sequence, $-^{+13}$AGGAAC^{+8}-, that resembles the consensus Shine-Dalgarno sequence -AGGAGG-. However, upstream of *lpxL1*, such a Shine-Dalgarno sequence could not be found. Also analysis of the −18 to +18 nucleotides around the ATG start codon applying the algorithm described by Kolaskar and Reddy (39), revealed that the *lpxL1* ATG start codon is inadequate. Thus, the absence of lipid A species carrying a 2OH C12 in wild-type *B. pertussis* may be explained by a poor translation initiation of *lpxL1* mRNA.

One of our initial goals was to obtain *B. pertussis* mutants in which the *lpxL* homologues, either separately, or as the complete operon, were knocked out. Because mutations in late acyl transferases have often been shown to reduce the toxicity of the LPS, we reasoned that *B. pertussis lpxL* mutant strains may form a good basis for the development of less reactogenic whole cell pertussis vaccines. The only viable mutant obtained was an *lpxL1* knockout. Because we were not able to isolate *lpxL2* or *lpxL1*/*lpxL2* mutants, *lpxL2* appears essential for *B. pertussis* viability.

Analysis of the *lpxL1* mutant strain did not reveal obvious differences as compared with the wild-type strain with respect to its growth characteristics, virulence factor expression profile, endotoxic activity, or LPS content, again indicating that *lpxL1*, under standard laboratory conditions, is not expressed. This situation is probably beneficial for the bacterium, because the hexa-acylated LPS species, as we showed for the LPS from the *lpxL1*-overexpressing strain (Fig. 6*A*), is highly active and would rapidly activate the host innate immune system and thereby evoke clearance of the bacterium. However, it is known that *B. pertussis* also has a partial intracellular life cycle (40). Inside a cell, a bacterium encounters a completely different environment, which implicates that the requirements to survive may also be different. It was previously shown that a late acyl transferase mutant of *N. gonorrhoeae* displayed a decreased survival inside urethral epithelial cells (37). In addition, Gibbons *et al.* (38) proposed a model in which lipid A-2-hydroxylation functions in the suppression of host cell signaling, permitting a more prolonged survival of bacteria in the host cell. Therefore, we hypothesized that the presence of an extra secondary 2OH acyl chain may be important during infection of eukaryotic cells by *B. pertussis*. Indeed, we showed that the recovery rate of the *lpxL1* mutant strain was much lower than that of the wild-type strain after infection of human macrophages. This was not due to a decreased association of the mutant strain to the macrophages, because analysis by flow cytometry showed that the mutant cells associated significantly better to the macrophages than did the wild-type strain. The reason for this increased association remains unknown but may reflect alterations in the LPS core domain, which could influence lectin-mediated adherence of the bacteria to the macrophages. Overall, our results suggest that the activity of LpxL1 is indeed required for an efficient infection of human macrophages and may therefore form an important factor for successful host infection by *B. pertussis*.

Acknowledgments—We thank Christian R. H. Raetz (Duke University, Durham, NC) for kindly providing E. coli strain MLK53 and Lucy Rutten (Utrecht University) for purified PagL.

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